The study of inheritance in bacteria has, for the most part, been confined to the investigation of mutational changes in the course of clonal reproduction. With the exception of experiments on pneumococcus type transformations there have been few studies on the direct hereditary interaction of one bacterial type with another. The conception that bacteria have no sexual mode of reproduction is widely entertained. This paper will be devoted to the presentation of evidence for the occurrence in a bacterium Escherichia coli, of a process of gene recombination, from which the existence of a sexual stage may be inferred.

The genic basis of microbial inheritance does not depend on the demonstrability of a sexual phase in bacteria. However, more powerful genetic methods paralleling classical Mendelian analysis would be available if it were possible to follow the inheritance of characters in the products of a sexual fusion. The few examples of this approach thus far reported have provided no incontrovertible evidence for sexual reproduction in bacteria.

The phenomenon of paragglutination in the colon-typhoid-dysentery group might be regarded as an instance of bacterial hybridization, and was so interpreted by Almquist (1924). As reported by numerous authors, paragglutination refers to the development of new types which react with antisera for each of two distinct strains, when these are grown together in mixed culture (Kuhn and Ebeling 1916; Salus 1925; Wollman and Wollman 1925). The significance of these observations has been attacked by several authors (Breinl 1921, Arkwright 1930, and Kauffmann 1941), chiefly on the grounds that the paragglutination represents a non-specific cross-reactivity characteristic of "rougher" phases of these organisms. Hansen (1929) failed to obtain paragglutination in her experiments. In the light

of more detailed recent information on the antigenic structure of this group, this problem certainly deserves a critical reinvestigation

Sherman and Wing (1937) have described experiments designed to detect recombinations of fermentative characters in mixed cultures of various E. coli and Aerobacter aerogenes strains. While new combinations of biochemical characters were found, similar types were found to an equal extent in pure cultures, so that these authors could not infer the occurrence of a sexual fusion. Their experiments are of the greatest interest, however, since they represent the first attempt to study this problem in bacteria by genetic methods using clearcut characters. Gowen and Lincoln (1942) later performed similar experiments with strains of Phytomonas stewartii using cultures differing in morphological and pigment characteristics. As in Sherman and Wing's studies, these authors were unable to differentiate the new types they found in their mixed cultures from types which arose spontaneously in single cultures. For this reason a definite conclusion could not be drawn from their results.

A discussion of hereditary processes in bacteria must take into account the extensive work on transformation of pneumococcal types, first described by Griffith (1928), and culminating in the isolation of the transorming principle in chemically characterizable form by Avery, MacLeod and McCarty (1944). These studies have revealed that under special experimental conditions, a product isolated from a serologically specific, smooth, pneumococcus culture will convert cells of a non-specific rough culture to the smooth type characteristic of the source of the transforming principle. So far as is known, such transformations can be performed in only one direction (rough to smooth) and only under very special conditions. Boivin (1947) has reported a similar transformation involving the somatic polysaccharide of a strain of E. coli. There have been

reported other instances of varying credibility (Kasarnowsky 1926; Lommel 1926; Legroux and Genevray 1933; Frobisher and Brown 1927; Burnet 1925; Holtman 1939, Cantacuzène and Bonciu 1926). These studies have a direct bearing on recombination experiments, since transformations of this sort might be responsible for the occurrence in mixed cultures of some new types which are interpretable as recombination types. This will be discussed in more detail later.

Morphologically unusual forms of various bacteria have been described by Mellon (1925) as zygospores, and been taken to imply sexual fusion. Smith (1944) and Dienes and Smith (1944) have suggest that the "Large Bodies" observed in some Bacteroides cultures might represent a sexual phase, and Dienes (1946) has made a similar interpretation of Large Bodies in Proteus. Star-shaped aggregations of Phytomonas tumefaciens have been studied by Stapp (1942), and more recently by Braun and Elrod (1947) and provoked all of these authors to the suggestion that the stars represented some sort of sexual fusion. As Luria (1947) has pointed out, however, "most of the older material presented in support of the hypothesis of sexuality in bacteria cannot be used as genetic evidence because of the lack of information on the exchange or recombination of discrete hereditary characters in the course of the supposed sexual fusion."

In the absence of zygote-segregation methods, the only techniques available for analysing the genetic structure of bacteria have been mutation analyses. The bacteriological literature is full of reports of bacterial variations, or so-called "dissociations" referring to the development within previously pure clones of new and distinctive types. While these are superficially very similar to the mutations occurring in higher organisms, many bacteriologists (e.g., Rahn, 1937) have rejected a mutational interpretation of bacterial variation, chiefly on the grounds that the variants are

often capable of reverting to the original form. This objection is without substance in view of the commonplace occurrence of reversion both in Drosophila, (Timofeef-Ressovsky 1937), and Neurospora, (Ryan and Lederberg 1946).

Another distinction that is deeply implanted in classical bacteriology is that the germinal material of bacteria is thought to be capable of direct adaptive modification in response to deleterious conditions, whereas this Lamarckistic viewpoint has now been virtually eradicated from the thinking of students of heredity in higher forms.

Proof that adaptive changes in bacterial populations are due to pre-occurring spontaneous mutations and their subsequent selection by the deleterious environment has not readily been attained. Luria and Delbrück (1943) have, however, studied the occurrence in E. coli of phage-resistant variants of which are detected by the application of a specific bacteriophage (T1) to a sensitive population. On the assumption that the resistant variants develop in response to the application of the phage, there should be no greater variation in the number of resistant cells which can be elicited by the phage in similar samples taken from a series of similar cultures than there would be in the case of similar samples taken from the same culture. On the other hand, it was predicted that there would be a wide variation in the number of mutants demonstrable in a series of separate but similar cultures if these mutants arise spontaneously previous to the application of the phage, which acts simply to demonstrate them. This increased variance arises from the fact that the occurrence of a mutation early in the growth of a culture will lead to the occurrence of a large clone of mutant descendants, whereas a mutation which fortuitously occurs later in the growth of the culture will have only a few mutant descendants.

Although an exact mathematical solution of the problem has not appeared, the variance found in Luria and Delbrück's experiments was much larger than could be explained on the direct adaptation hypothesis, and was in good accord with the predictions of an approximate mathematical theory which they developed.

In a similar way, it has been shown that a number of "adaptive" changes in bacteria are the result of spontaneous mutations occurring previous to the application of the agent used to select them. These include, in addition to mutation of E. coli to phage resistance as already discussed, resistance to additional phages, (Demerec and Fano, 1945), resistance of Staphylococcus to penicillin (Demerec, 1945) and sulfonamides (Oakberg and Luria, 1947), resistance to radiation in E. coli (Witkin, 1947), and nutritional adaptations of Clostridium septicum (Ryan, et al 1946) and of mutants of E. coli (Ryan and Lederberg, unpublished.) Recently published examples of presumably direct adaptive mutations either have not excluded entirely the possibility of previous mutation and selection, as in the adaptation of yeast to pantothenate synthesis, (Lindegren and Raut 1947) or have not full demonstrated the heritable character of the modification, as in the adaptation of E. coli to resistance to 2-chloro-p-aminobenzoic acid. (Stranskov, 1947). One may conclude, then, that as in the higher organisms, the germinal determinants of bacteria are not in direct adaptive rapport with the environment.

Additional evidence of the overall similarity of bacterial genes to those of other organisms is contained in the experiments on the induction of phage-resistance mutations by Demerec and Latarjet (1946). They reported that X-rays induced mutations of E. coli to T1-resistance, and that the mutations induced were linearly proportional to the X-ray dose. The linear type of response furnishes strong support, on the basis of the "hit-theory" for the

occurrence of a single localized determinant in the cell whose inactivation leads to the mutation.

Finally, by the use of X-rays, and other mutagens, Tatum (1946) has obtained nutritional mutants of E. coli which are in every way analogous to such mutants in Neurospora where their analysis by orthodox genetic methods has shown them to be effects on single genes. Other investigators have produced similar mutations in E. coli (Roepke, et al, 1944) and other bacteria (Burkholder and Giles, 1947; Gray and Tatum, 1944). The mutants obtained by Tatum have subsequently been utilized to demonstrate factor recombination, as will be described in this dissertation.

Materials and Methods

The strain selected for these experiments is Escherichia coli, K-12. It was originally isolated from human feces a number of years ago, and has been carried on agar slants at Stanford University since that time. It was used there as a typical E. coli for demonstrations in student laboratories. It has been found to ferment lactose, to produce indole, and to be susceptible to each of the E. coli phages, T1 to T7, collected by Demerec and Fano (1945). Since it ferments sucrose very slowly, if at all, it would be classified as Escherichia coli "commune". It is moderately motile as determined by hanging-drop observations, and by its slow movement through semi-solid gelatine-agar.

Mutant strains of E. coli characterized by specific growth factor requirements have been obtained after treatment with x-rays, ultra-violet light and nitrogen-mustard. Such strains have been described by Tatum (1945, 1946) and by Roepke (1944). A single nutritional requirement is established at a single mutational step, and on the basis of studies on Neurospora is regarded as based on a change in a single gene. By successive treatments, multiple mutant strains with several genetically and biochemically independent nutritional requirements have been produced. The strains used in these experiments are described in Table 1. In general, the nutritional characteristics of a strain are ascertained by inoculating media consisting of the basal medium plus various supplements; lack of visible growth in the absence of a given growth factor and optimal growth in its presence are the criteria for the determination of the nutritional requirements of a strain.

A mutant strain can be signified by suffixing a "-" sign to the initial of the substance in question; e. g. B-Pa-C- refers to a strain which is deficient in the synthesis of biotin,

phenylalanine and cystine. On the other hand particular emphasis can be placed on the ability of a strain to synthesize a particular growth factor by suffixing a "+". Thus B-Pa-C-T+L+B_1+ would refer to a strain deficient in the three factors mentioned above, but capable of growth in the absence of threonine, leucine, or of thiamin. The representation of a growth factor requirement by a minus sign is justified by the a priori consideration that a mutation establishing a growth factor requirement generally represents the loss of a function, and by the experimental finding of Beadle and Coonradt (1944) that wild are dominant to mutant genes in Neurospora heterocaryons. Strains which are "+" for all growth factors have been called prototrophs (Ryan and Lederberg, 1946) since this is the nutritional condition of the parental wild type E. coli strain from which all the mutants were ultimately derived.

Mutations for resistance to specific bacteriophages or bacterial viruses have proven to be exceedingly useful. They are readily obtained as spontaneous mutants by plating a large number of sensitive bacteria with the particular virus in question; only resistant mutants escape lysis and may be recovered as "secondary" colonies (Fig. 1). Resistant mutants are readily freed from residual virus by serial single colony isolation. Resistance to a given virus may be scored by streaking a loopful of bacteria on an EMB or nutrient agar plate at right angles to a previous streak of the virus suspension (Fig. 1).

It has been found, however, that mutations for resistance to a given virus are not entirely specific, but that resistant mutants display "cross-resistance", i. e., are also resistant to other viruses, Demerec and Fano 1945). For example, most T1-resistant types are also resistant to T5 (For the nomenclature of the bacterial viruses used in this investigation, and a detailed

account of the cross-resistance patterns of another strain of E. coli B, see Demerec and Fano (1945)/. The cross-resistance patterns of K-12 are similar to those of E. coli B with the exception that T1-resistant mutants which are sensitive to T5 are not tryptophaneless, as have been reported by Anderson (1946) for the corresponding mutants of E. coli B. In this paper, the designation V_1^r will be used for the more frequent T1-resistant mutant, which is also resistant to T5.

The specificity of fermentation reactions of various bacterial species is a clear indication that the ability to ferment certain sugars has a distinct genetic basis. Because they would be so easily scored on indicator media, mutants unable to ferment various sugars have been looked for. Particular attention was paid to the possibility of isolating "lactose-negative" or "Lac-" mutants, because of the taxonomic significance which has been attached to this character.

The detection of fermentation mutants is readily accomplished by the use of indicator media. The medium "EMB-lactose" used in routine bacteriological work was found to be highly useful. It consists of the following: in G./l., Peptone (or "N-Z-Case") 10, Yeast Extract 1, lactose 10, agar 15, Eosin Y 0.4, Methylene Blue 0.06, sodium chloride 5, dipotassium phosphate 2. On this medium, colonies of bacteria which can ferment lactose (or any other sugar added in its place) rapidly turn a deep purple color, while colonies of non-fermenting organisms remain white or pink but may slowly turn light blue.

Lac-mutations have been recovered in two instances. Among 15,000 colonies of strain Y-10 (T-L-B_1-) obtained by spreading a culture previously treated with ultraviolet light, on EMB-lactose agar, a single pink colony was noted. It proved to be the same,

nutritionally, as Y-10 and was therefore regarded as a Lac-mutant and not a contaminant, this stock is labelled Y-53. Among 30,000 colonies of Y-40 (B-M-V_1^r) a single Lac- was recovered following treatment with nitrogen-mustard (Tatum, 1946) and was designated as Y-87. Tests showing that these independent mutations are probably allelic will be described in a later section (see Table 4g). Strains Y-53 and Y-87 differ in the rate at which the Lac-character reverts to the Lac+ condition, but whether this is due to different allelic states or to differences at other loci, cannot be definitely asserted.

Prelimary attempts to obtain maltose, mannitol, and galactose-negative mutants were not successful, presumably because the populations tested were too small. A glycerol-negative strain has been obtained, but the wild type ferments this polyalcohol so poorly to begin with that accurate scoring is difficult; studies in this character have, therefore, not been pursued.

It is particularly fortunate that resistance-tests can be conducted on EMB agar, since this allows the characterization of a strain with respect to virus-resistance and to lactose-fermentation with a single streaking (see Fig. 1).

Morphological variation has occasionally been noted (exceedingly rough or very mucoid colonial form) but is relatively unsuitable for genetic work because the presumably random choice of prototroph recombinants may be influenced.

In additon to the EMB agar already described, a number of other natural or "complete" media have been used. The Difco product "Penassay Broth" has been used most extensively, and is satisfactory for the preparation of inocula, except that it must be supplemented with cystine for the growth of cystineless organisms, such as strain Y-24. Other satisfactory media include a broth consisting of: peptone 5, glucose 5, yeast extract 3, g./l, as well as Difco Nutrient

Broth, and diverse concoctions containing peptone or casein hydrolysates and meat or yeast extract.

The synthetic or minimal medium contains, in g./l.,: NH_4Cl 5, NH_4NO_3 1, Na_2SO_4 2, K_2HPO_4 3, KH_2PO_4 1, glucose 5, asparagine 1.5, $MgSO_4$ 0.1, trace elements (Beadle and Tatum 1945) and $CaCl_2$, a trace.

To avoid flocculation when used with agar, the glucose and agar in solution should be autoclaved separately, and mixed with the other components just before using. Unwashed agar (Difco) is sufficiently free of the growth factors under consideration to be satisfactory for many experiments; the use of washed agar, however, is recommended for the cleanest results.

The detection of recombinants is based upon the inability of biochemical mutant bacteria to proliferate in the absence of their specific growth substances. Plating in minimal agar, therefore, has the effect of a sieve for prototroph cells. To insure against contamination with prototrophs derived by reverse mutation which has been noticed at certain loci, it has been desirable to use multiple biochemical mutants as the parental stocks in recombination studies. Coincidental reversion at two or more loci is ~~the~~ theoretically improbable, and experimentally undemonstrable (see below). For example, plating either B-M-T+L+B_1+ or B+M+T-L+B_1- separately into nominal agar did not lead to the appearance of prototrophs, B+M+T+L+B_1+. When, however, a mixture of these cell types was so "sieved", one prototroph was found for ca. each 10^7 cells inoculated. These have been assumed to arise from the recombination of "+" alleles to form the prototroph.

In early experiments, the two multiple mutants were inoculated together into a complete medium and allowed to grow in mixed culture before plating into minimal agar. This method is not entirely satisfactory because it allows possible selective differentials to alter the relative frequencies of different recomination classes. A modified

procedure was developed, which will now be described in detail.

The mutant stocks are maintained on "complete" agar slants, transferred at intervals of 6-8 weeks. They are inoculated separately into test-tubes containing about 10 ml. of liquid complete medium and incubated overnight at 30° C. with gentle shaking. The following morning, an additional 10 ml. of the same medium is added to each culture, and the tubes are incubated in the same manner for an additional 3-5 hours. These cultures contain from 1-4 x 10^9 cells per ml. They are then washed in the following manner: the cotton plugs are replaced with sterile corks which have been kept in 95% alcohol and the alcohol flamed off just before using. The cultures are then centrifuged at ca. 2500 r.p.m. for 20 minutes, which suffices to pack the cells in the bottom of the test tubes. The supernatant medium is carefully poured off, and the tube is rinsed with ca. 10 ml. sterile distilled water, care being taken not to disturb the pellet. The cells are then resuspended in an additional 15-20 ml. sterile water, and recentrifuged. The supernatant wash water is decanted and replaced with an equal volume of fresh sterile water, in which the cells are suspended. In the meantime, minimal agar plates are prepared. A bottom layer of ca. 15 ml. minimal agar is poured into each Petri plate and allowed to solidify. Cell-suspensions of different mutant stocks are mixed at this time and measured quantities (usually ca. 10^8 - 10^9 cells) are pipetted onto the agar surface. At this time also, one may add such growth factor supplements as are desired to permit the growth of recombination types other than prototrophs. The cell suspensions are then mixed into a layer of ca 10 ml. molten minimal agar (at ca.45-50° C.) which is poured onto the plates. After the agar has hardened, the plates are incubated at 30° C. for a period of 48 hours. At this time recombinant prototroph colonies will be found distributed throughout the plate, many of them at or near the surface and accessible to picking

for further characterization.

The procedure may be varied in several ways. It is important, however, that the inoculum consist of "young" cells, since cultures of 24 hours or older have given quite inconsistent results. It is possible to store the inoculum in distilled water for at least twenty-four hours without appreciably affecting the yield, which suggests that the aggregation of genetics leading to the recombination process occurs in the molten or in the solidified agar. This occurrence must, however, take place within a few hours, since the recombinant prototrophs are not appreciably slower to appear than wild type cells in a similar physiological state which are streaked on the surface of the plates. Presumably therefore one could increase the yield of prototrophs by making conditions more favorable for the free contact of the cells; as by packing them together in a centrifuge tube in minimal liquid medium. However, the complication of proliferation of prototrophs already formed would interfere with the interpretation of such an experiment. Many physiological factors may interfere with the recombination process, and, for example, the yield may be reduced markedly by inoculating too heavily, or by omitting an under-layer of agar into which, presumably, deleterious metabolic products may diffuse. Instead of mixing the cells in semisolid agar, it is possible to streak the mixture on the surface of slightly dried minimal agar plates. Under these conditions, however, the prototroph colonies are likely to be more heavily contaminated with the residual parental mutant types.

For most purposes, however, this contamination may be ignored, as will be shown in a later section. Prototroph colonies are, then fished and streaked directly on EMB plates, or otherwise tested, to classify them with respect to other factors that may be segregating.

Experimental Results. - Spontaneous mutations of bacteria in pure culture were studied as a preliminary to the investigation of recombi-

nation. The overall frequency of random biochemical mutations in untreated cultures is less than 0.1% (Tatum 1946) although samples totalling not more than 5,000 cells have been studied so that the precision of this measurement is doubtful. In view of the low rate and sporadic, independent occurrence of such spontaneous mutations, however, they may be regarded as a negligible factor in this study.

The spontaneous reversion of biochemical mutants to prototrophs is under continuing study (Ryan and Lederberg) and will be reported on more fully elsewhere. It has been found that many biochemical mutants of E. coli, K-12, will revert at a low rate, prototrophs being found in the proportion of 10^{-7} in 24-hour cultures of single mutants. Reversions of different factors are, so far as has been yet ascertained, entirely independent; as predicted from the low rate of reversion of the individual factors, in ca. 10^{-10} cells examined no instance was found where reversion had occurred at both loci of a double mutant. Such a coincidence would have led to the appearance of a prototroph in a culture inoculated with a double mutant such as T-L-. On the basis of these considerations, only double and triple mutants have been used in the study of recombination.

The frequency of spontaneous mutations to virus resistance has the same low order of magnitude as nutritional reversion (Luria and Delbrück, 1943). Mutations from resistance (V_1^r) to susceptibility (V_1^s) have not been described, owing to the lack of efficient techniques for the detection of such reversions.

Prototroph Recombination Types: Since coincidental spontaneous reversion at two or more loci does not occur at a sufficiently high rate to be detected, the presence of prototrophs in mixed cultures of multiple mutants is evidence for gene recombination. Each mutant is capable of synthesizing all the growth factors for which it is not deficient; therefore, different mutants should have "+" alleles for all but the

two or three mutant genes that characterize each strain. The segregation of prototrophic alleles of every gene into one cell would result in a prototrophic cell. It would develop into a visible colony on minimal medium while other mutant cells would be unable to proliferate due to the absence in minimal medium of their nutritional requirements.

When washed samples of mixed cultures of B-M-P+T+and B+M+P-T- were plated into minimal medium, about 100 colonies developed for each billion (10^9) cells inoculated. No colonies appeared after innoculation of samples from the individual double mutants. One interpretation of the occurrence of prototrophs, designated as B+M+P+T+, is that the P+ and T+ genes of B-M-P+T+ and the B+ and M+ genes of B+M+P-T- have segregated into the same cell. This is a recombination hypothesis; alternatives will be discussed in the next section.

The possibility must be considered that the prototrophs do not consist of some sort of association of the unaltered mutants. In a classical illustration of nutritional symbiosis, since designated as syntrophism (Lederberg 1946), Valentine and Rivers (1927) showed that Hemophilus canis and H. parainfluenzae, which require X and V factor respectively, would grow in mixed culture in media lacking these substances. They concluded that these growth factors, synthesized by the individual bacteria, were exchanged via the medium. While there is no good reason to doubt this conclusion, these authors did not, in fact, conclusively demonstrate that this was the mechanism of the interaction. It is possible that cells were present in their mixed cultures which, as a result of gene recombination, required neither of the two factors. The situation is obscured by the use by these authors of serial transfers of large numbers of bacteria.

Syntrophism has been shown to occur with E. coli mutants (Lederberg 1946) (Lampen et al, 1947). It is not likely, however,

that it plays a significant role in the appearance of prototrophic colonies. Washed cells inoculated into minimal medium do not show syntrophism until small quantites of their required growth factors are added. In minimal agar plates heavily inoculated with a washed mixed culture a uniform turbidity does appear which is ascribable to a limited exchange of factors and subsequent syntrophic growth.

Evidence of several sorts has been obtained for the homogeneity and uniqueness of prototrophs isolated from mixed cultures. Most significant, they are quite stable and attemps to detect the original mutants in recombination prototroph cultures by an efficient selective technique (Lederberg and Tatum 1946a, o,) have been unsuccessful. Massive doses of ultra-violet light, killing all out 10^{-5} of the cells in the culture, were no more successful in breaking up the supposed associations. In addition, prototrophs obtained from B-M-P+T+V_1^r and B+M+P-T-V_1^s were studied. Both susceptible and resistant cultures were obtained. Although one of the parental strains is resistant, the susceptible cultures were uniformly lysed upon application of the phage; on the other hand, there was no change in the nutritional behavior of cultures of resistant prototrophs subsequent to the application of the virus, which would be expected, in an association of the original mutants, to lyse the susceptible B+M+P-T-V_1^s cells and leave only B-M-P+T+V_1^r.

A nicotinicless mutant has been obtained by ultra-violet irradiation of a prototroph derived from P-T- and B-M-. The prototroph in which this mutation occurred could have been neither a heterocaryon nor an association of diverse types, since in either case the absence of nic+ genes in the mutant would require the simultaneous mutation of more than one representative of these gene. This coincidence is highly improbable. The microscopic examination of seeded agar supported the conclusion that the cells of strain

K-12 are well dispersed, so that most of the colonies that appear would be derived from single cells when only a few hundred cells are inoculated per plate, as was done subsequent to the initial isolation of prototrophs. Single cell isolations from a "recombination prototroph" strain have been made by Dr. M. Zelle of the National Institute of Health; all of the single-cell cultures tested were of the same nutritional and virus-resistance type as the culture from which they were isolated. Finally, the diversity of recombination types described below is incompatible with the hypothesis that they result from a simple combination of cells, or

Transformation, preliminary expts: The evidence just presented points to the conclusion that the prototrophs are a new type of cell, which did not arise by spontaneous changes in a single double-mutant strain. Gene recombination, which was postulated above, is however, not the only interpretation for the origin of these new types which would fit the evidence that has been presented. By analogy with the systems which have been described in pneumococci (Avery et al, 1944) and other strains of E. coli (Boivin 1947) one might postulate that genotypically distinct cells interact not through cell fusion, but through the release of "transforming substances" diffusing through the medium. Such transforming substances would have the property of inducing or directing mutational changes in the cell receiving them so as to lead to what appear to be recombination types.

Since the conditions of the recombination experiments require that any transforming substance be present in the medium, an attempt was made to modify a nutritional mutant with a culture filtrate from another mutant. B+M+P-T-V_1^S was grown in YB broth, and samples of 12- and 36- hour cultures were freed of cells by centrifugation and filtration through an ultra-fine sintered glass filter. The filtrate was diluted with an equal volume of YB and inoculated with

B-M-P+T+V_1^r. As a control, B-M-P+T+V_1^r cells were inoculated with B+M+P-T-V_1^s into filtrate broth. After the cultures were incubated for 48 hours, they were analyzed for prototrophs by the methods described above. None were found in the B-M-P+T+V_1^s cultures grown in the presence of B+M+P-T-V_1^r filtrate, indicating the absence of an active transforming principle in the medium under these conditions. On the other hand, the growth in mixed culture of B-M-P+T+V_1^r and B+M+P-T-V_1^s cells resulted in the appearance of numerous prototrophs.

Additional attempts were made to determine whether "transforming activity" could be separated from the living cell. This is tantamount to replacing one of the parental cell types in a recombination experiment with an extract prepared from it, or with comparable materials. Conditions comparable to the plating described on p.12 were used, as well as cell extracts prepared by Boivin's method. (Boivin, 1947).

No activity was found in supernatants of suspensions of Y40 or Y53, together or separately, as tested by plating the supernatants with Y40 or with Y53, into minimal medium and looking for prototrophs. The only manipulation involved here consists of the removal of most of the bacteria from suspension in minimal liquid medium, in which they had been allowed to remain for varying periods up to six hours. "Activity" remained in association with the cells, as tested by plating them with the alternate type. Equally negative results characterized attempts to reveal transforming activity in culture filtrates and cell autolysates prepared, as mentioned, according to Boivin (1947).

Finally, the addition of desoxyribonuclease, (kindly provided by Dr. M. McCarty) in a final concentration of .05 mg./ml. to the mixing and plating medium had no effect on the number of prototrophs which appeared on "crossing" Y40 and Y53.

Additional experiments and considerations of a purely genetic character will be described below; at this point in the experiments, the interpretation of prototrophs as recombination types resulting from a sexual process was adopted as a working hypothesis, and further experiments were designed to elucidate it in detail.

Other recombination types.

If prototrophs arise from the segregation into the same cell of + alleles from its sexual parents, there might, (in a haploid system such as E. coli might well be thought, a priori, to represent,) be found in the same mixed cultures other combinations involving - as well as + alleles. In the first attempts to detect addition segregation types the cultures Y24, B-Pa-C-T+L+B_1+V_1^s and Y 46, B+Pa+C+T-L-B_1-V_1^r were used. There were, thus, available 7 markers some of which might be expected to segregate from the others, and give rise to a variety of recombination types.

Unfortunately, it would not be possible to detect all of the 2^7 or 128 possible recombination types. Only those types could be detected which would grow in a medium in which both of the parents would be suppressed. That is, either biotin, phenylalanine or cystine would have to be omitted to keep Y24 from predominating in the plates, and either threonine, leucine or thiamin to suppress Y46. In fact, it would be preferable to omit at least two factors required by each parent in order that "contamination" by back-mutants at a single locus be eliminated. While this restricts the number of recombination classes that could be isolated, it still leaves a great many. Four markers (two of the - alleles of each parent) would be used up in order to detect the "prototroph" recombinations, but the other three would be free to segregate in such combinations as the genetic system determines.

Since the V_1 locus has not been used for detecting

recombinants, there are a total of nine nutritional double-requirement types - B-T-; B-L-; B-B_1-; Pa-T-; Pa-L; Pa-B_1-; C-T-; C-L-; C-B_1- which could be detected, as well as six single-requirement types and prototrophs. Such types would be found by plating mixtures of Y24 and Y46 into minimal agar containing two supplements such as biotin and threonine. On this medium, both parents would be suppressed, but the recombinant (ex hypothesi) types : B-T-; B-; T-; and prototrophs should be able to grow. Colonies isolated from such a plate would possibly be any of these four classes, and must be classified more fully. This was accomplished by fishing them into small tubes of sterile water, and taking small inocula into a series of tubes of minimal medium supplemented with threonine, with biotin, with neither and with both. The inability of a culture to grow in the absence of a growth factor indicates the - allele, while the tube containing both biotin and threonine serves as a control. Prototrophs, of course, will grow on each of these four media; B- only on the media containing biotin, T- similarly, and B-T- only in the doubly supplemented tube.

The result of such an experiment is summarized in Table 2. In order to determine the proportions of the various types, the number of prototrophs was used as a standard. The total number of prototrophs obtained from those plates which had supplements allowing the development of a given type was compared with the total number of that type isolated. The relative frequency of prototrophs and B_1-, for example, was found using plates supplemented with thiamin, with thiamin and biotin, thiamin and phenylalanine, and thiamin and cystine. The growth requirements of the more interesting "double-requirement" segregants were checked several times using 10 ml. volumes of medium , and isolates purified by serial single colony isolation. V_1 was, of course, also segregating, but was scored only for some of the thiaminless and prototroph isolates.

As indicated by the table (Table 2) several different recombination types were found. For example, considering the factors B and B_1 only, it will be recalled that the parental arrangements are B-B_1+ and B+-B_1- in Y24 and Y46 respectively. In addition to the parental arrangements in such recombinants as B_1- (B+) and B- (B_1+), the non-parental combinations are found in such types as prototrophs (B+B_1+...) and B-B_1-. However, since these can only be detected in the uniform recombination class Pa+C+T+L+ the four types mentioned are not strictly complementary to each other, and comparison of the frequency is not particularly meaningful, as it would be if the cross were simply of the form $\underline{xy}$ x $\underline{XY}$, where xY and Xy should be equally frequent

Beyond the mere existence or many of these types, it will be noted that there are considerable differences in their relative frequencies. However, too much weight cannot be placed on these discrepancies since, in this experiment, the two parental types are grown together in mixed culture in "complete" medium for 48 hours before being washed and plated. Different recombination types formed during growth in liquid might be subject to selective growth differentials which would alter their relative frequencies. It was to counter this objection that the modified procedure described on p.12 was developed.

Although nutritional requirements are not objectionable as markers, and are indispensable for the detection of recombinants, it was considered that such markers as virus resistance and sugar fermentations might be more readily manipulated and scored in large numbers. For further study of segregations, therefore, the behavior of the factors Lac and V_1 have been especially scrutinized.

The character $V1^r$ has been particularly useful because a selective procedure exists by means of which it can be introduced

mutationally into any desired gene combination. The application of a "cross"heterozygous at this locus to the demonstration that prototrophs are not simply cell-associations has already been mentioned. In the course of those tests, 10 prototrophs each were isolated from the crosses B-M-P+T+V_1^r x B+M+T-P-V_1^s, and B-M-P+T+V_1^s x B+M+P-T-V_1^r respectively. In the first case, 8 were V_1^r while 2 were V_1^s. In the second, "reversed" cross, 3 were V_1^r while 7 were V_1^s. The apparent reversal of ratios in reversed crosses, in this small sample, suggested a technique by which the basis of the non-random distribution of recombination classes might be examined. The observations were extended, therefore, to collect more data for this cross and to study other combinations as well.

The segregation of V_1 alleles into prototrophs resulting from three different sets of mutant combinations in which the parents were heterozygous for this locus is shown in table 3. It will be noted immediately that there is a large discrepancy between the frequency with which prototrophs are V_1^r or V_1^s as a result of the "reversal" of the parents in which these alleles are introduced. This discrepancy amounts to a $X^2 = 199$ (for three degrees of freedom) when the values are cumulated. On the other hand comparisons made between the results of "reversed"crosses, the ratios being similarly reversed, show a fairly good fit, a cumulative $X^2 = 9.8$ (for three degrees of freedom) $p = .02$) being obtained. These tests illustrate the combinatorial or Mendelian character of inheritance whereby "gametic frequencies are invariant in respect of any gene substitution applied systematically to the genic content of an organism and of the gametes it produces." (Fisher, 1947) A value of $p = .02$ for goodness of fit under the hypothesis of Mendelian behavior is not as reassuring as one would like, but may perhaps be ascribed to errors in scoring V_1 rather than to a real deviation from the theory. There can, at any rate,

be no doubt as to which of the two modes of comparison gives the better fit.

Subsequent to the completion of the experiments of Table 3, the Lac-mutant stocks Y53 (T-L-B_1-Lac-) and Y87 (B-M-V_1^r Lac_2-) were obtained as already described. In addition, a V_1^r mutant of Y53, Y64, (T-L-B_1-Lac-V_1^r) was readily obtained. The development of these stocks permitted a more critical experiment similar to those summarized in Table 3, but in which the segregations of two factors from various parental combinations could be readily studied. In tables 4, the data from a number of individual experiments are recorded, and analysed for their homogeneity. In tables 5 and 6 the data are summarized, and the results of the different crosses are compared much as in table 4.

Inasmuch as the segregation of B_1+/B_1- was also used in this experiment, a word will have to be said concerning this locus, in anticipation of a further discussion below. It was noted in crosses of B-M-T+L+B_1+ x B+M+T-L-B_1- that the yield was approximately ten times as great on thiamin-supplemented as on minimal agar, indicating a B_1-/B_1+ ratio of about 10;1. Rather than separate the 10% B_1+ colonies from the total found on thiamin-supplemented plates, the expedient of comparing the segregations of Lac and V in colonies from minimal and from thiamin-supplemented plates was used. In the latter, B_1- types would be so preponderant that any appreciable deviation from the B_1+ segregation frequencies should be noted among these colonies despite their "contamination" with 10% B_1+.

The significant heterogeneity of the data of Tables 5 and 6 is rather disturbing, but no method has yet been found of avoiding it, and its extent and character are such as to make very laborious any attempts in this direction. The null hypothesis of table 6 must be modified accordingly. It should read: "that the deviations

between the experiments of Table 4 are not greater than can be counted by their intrinsic heterogeneity."

An attempt was made to deal with this problem quantitatively by the variance-ratio method. The X^2 for the various sets of experimental data are given in the tables. The variance of Table 4a is, however, very different from that of the others, and none is available for 4e and 4f as these were the results of single, large experiments. It has not, therefore, been possible to select a characteristic variance for the error or heterogeneity of the data by means of which to test the deviations between the three main experiments. The shift in the type of the rarest class with change in the parental couplings is, however, very striking and affords the clearest qualitative verification of the principle of mechanical recombination.

The conclusions which may be drawn from the analysis are:

1. A far better agreement with a Mendelian hypothesis than with a direct comparison of class frequencies in alternated crosses.

2. No difference in the segregations of Lac and V_1 in the B_1- as against the B_1+ progeny.

3. Non-random segregation of Lac and of V_1 with respect to the nutritional factors, as indicated by deviations from 1:1 ratios of alleles in the prototrophs.

4. Interaction between Lac and V_1 themselves. In this case, for example, the ratio of Lac $\underline{x}$: Lac $\underline{x}'$ is 1389:817 = 1.7 in the subclass V_1 $\underline{y}'$, but is 828:63 = 13.1 in the subclass V_1 $\underline{y}$. (Table 6).

In addition, experiments are recorded in these tables (4g) indicating that the separately obtained Lac- and the V_1^r mutations used in the experiments are indeed allelic, i.e., that no Lac+ or V_1^s segregants, respectively, occur among the progeny of crosses homozygous for Lac- or for $V1^r$.

The simplest combinatorial mechanism known is that of random or independent recombination. Since the data do not support this hypothesis, one is led to try the next most simple, and the one characteristic of all other organisms studied: the organization of genes into linkage groups, presumably corresponding to chromosomes.

The first problem which must be solved is: "How many linkage groups can be identified?" The data already presented are not sufficient; the behavior of the various nutritional markers themselves must be studied first.

If one of the five markers in the cross B-M-T+L+B_1+ x B+M+ T-L-B_1- were independent of the others, we might predict that the class "x"- would be equally frequent with "x"+ in the detectable recombination classes where all the other markers were+ . That is to say, either B-, M-, T-, L- or B_1-, depending on which ones were segregating independently, would occur in a 1:1 ratio with prototrophs.

To test this hypothesis, mixtures of these parental types were plated into agar medium supplemented with one of the five growth factors involved: biotin, methionine, threonine, leucine, or thiamin. On the biotin-supplemented plates for example, the two recombination classes: B-M+T+L+B_1+ and B+M+T+L+B_1+ are capable of forming colonies. If B is independent, they should be equal in number; if not, there should be a discrepancy from a 1:1 segregation. Colonies were, therefore, picked from such plates and scored by testing them on liquid medium for their growth requirements. The results are in Table 7.

Unfortunately, the B-M- parental type was not entirely suppressed when heavily inoculated into methionine-supplemented plates, due to a low level of contamination with biotin either or the agar, the methionine, or the cell suspensions. This resulted in the crowding out of any recombinants which were formed, so that figures

for the M-:M+ ratio cannot be given. Fortunately, this data turns out not to be critical, as noted below.

It will be seen from Table 7 that none of the other markers have segregated at random, B_1- being more frequent, and the others less frequent than prototroph recombinants.

To account for B- being less frequent than B+ in the M+T+L+B_1+ class, we may postulate that B is linked to one or more of these markers. Comparing the parental couplings: B+ with M+, T-, L-,B_1- we may conclude that it must be with M that B is linked.

Similarly, we note that B_1- is more frequent than B_1+. The parental couplings are B_1- with B+, M+, T-, L-. Therefore, B_1 must be linked with B or with M, or since these are linked in turn, with both of them. The linkage group: (B_1:B;M) therefore appears, although at this stage the relative order is not determined.

Both L- and T- are rarer than prototrophs. They must therefore be linked with each other, since the parental couplings are: T+ with L+(B_1+;B-;M-), L+ with (B_1+;B-M-), T+ respectively. We are not entitled to infer that the groups (B-;B;M) and (T;L) are or are not independent of each other since an interchange between them is necessary, although insufficient, to produce the detectable prototroph class. With this reservation, the map of Table 6 may be written, for these factors.

The linkage relations of Lac and V may be gotten from Table 6. In the cross (B_1-;B+M+)(T-L-)Lac- X (B_1+;B-M-)(T+L+)Lac+, it may be seen that Lac- is the more frequent, both in the B_1-B+M+T+L+ and the B_1+B+M+T+L+ classes. Lac, therefore, is linked with the (B_1-;B;M;) group although probably not closely with B_1 inasmuch as the B_1 and Lac segregations do not interact. We may, then write: (B_1;B,M;Lac).